Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med 2011;364:2119-27.

Supplementary Appendix

Methods

Patients

Inclusion criteria included measurable disease by CT, MRI or physical examination, ECOG performance status of 0 or 1, serum creatinine less than or equal to 1.6 mg/dL, total bilirubin less than or equal to1.6 mg/dL, WBC greater than or equal to 3,000 per mm^3, platelet count greater than or equal to 90,000 per mm^3, and serum AST/ALT less than 3 times upper limits of normal. Exclusion criteria included ocular or mucosal melanoma, prior high-dose IL-2 treatment (greater than or equal to 600,000 IU/kg per dose), previous gp100 peptide vaccines and a history of brain metastases. Patients with pulmonary function tests (FEV1) less than 65% of predicted, or who were immune compromised or receiving steroids were also excluded. All patients 50 years of age and older underwent cardiac stress testing to rule out reversible coronary ischemia. Twenty one institutions enrolled patients in this clinical study. All institutions had local IRB approval. Central IRB oversight was maintained initially by the NCI in Bethesda Maryland and subsequently by the IRB at Indiana University Health Goshen in Goshen Indiana. All patients gave written informed consent to participate in the trial. Central monitoring of the trial was done by a Data Safety Monitoring Board composed of a medical oncologist, a bioethicist, a biostatistician and a cancer survivor.

Response assessment

A partial response (PR) was defined as a 50% or greater decrease in the sum of products of perpendicular diameters of measurable lesions; no new lesions could appear, and none could grow by 25% or more. A complete response (CR) was defined as the complete disappearance of all evaluable lesions. All responses needed to be sustained at least 4 weeks to be recorded as a response. Blinded central review of all radiographic assessments reported to have SD for at least 3 months, PR, or CR was done by one radiologist at NCI. Progression free survival and overall survival were measured from the time of randomization. Progression of disease and death from any cause were defined as progression free survival events.

<u>In-vitro studies</u>

Patients underwent phlebotomy for immune monitoring studies prior to the start of treatment, as well as after completing every 2 cycles of therapy. PBMC and serum were cryopreserved for later study.

Intracellular staining for foxp3: Peripheral blood mononuclear cells (PBMC) were isolated on a ficoll gradient and stained for foxp3 as previously described[1]. In brief, cells were stained with anti CD4-FITC and anti CD8-APC antibodies, fixed and permeabilized, stained for intracellular foxp3 using the PE anti-human foxp3 antibody (eBioscience cat # 72-5776) and analyzed by flow cytometry.

In vitro sensitization assay: In vitro sensitization assays were performed as previously described[2]. In brief, PBMC were cultured with 1micromolar peptide and 300IU/ml IL-2 for 11-13 days and tested for reactivity by measuring gamma-interferon release after overnight coculture with peptide pulsed T2 cells. A positive assay was defined as greater than 100pg/ml gamma-interferon release and at least twice the release of pretreatment PBMC and all control peptides.

References for Methods

1. Ahmadzadeh M, Felipe-Silva A, Heemskerk B et al. FOXP3 expression accurately defines the population of intratumoral regulatory T cells that selectively accumulate in metastatic melanoma lesions. Blood 2008; 112(13):4953-4960.
2. Rosenberg SA, Yang JC, Schwartzentruber DJ et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nature Medicine 1998; 4(3):321-327.

<u>In-vitro results</u>

Immunologic analysis utilizing a sensitive 12 day in vitro sensitization assay was performed on samples available from patients on both cohorts to determine the presence of PBMC reactive with the gp100:209-217 (210M) peptide in samples obtained before and after 4 cycles of treatment. Samples were analyzed following 4 vaccinations (as opposed to 2) because we hypothesized that repeated vaccination would be more likely to induce vaccine specific cells. This time point potentially introduced a bias of enrichment of samples from patients that were responding to treatment, on both arms of the study. Twelve post-treatment samples were available from patients on the IL-2 arm and none developed reactivity against the immunizing peptide. Samples were available from 37 patients in the IL-2 plus vaccine arm and 7 developed anti-peptide reactivity (4 of 11 patients with objective clinical responses compared to 3 of 26 non-responding patients, p=0.16). Thus, there was no relationship between the development of anti-peptide reactivity and objective clinical response.

Because of the ability of IL-2 administration to mediate the growth of $CD4^+$ $foxp3^+$ T regulatory cells, we analyzed the percent of these cells in PBMC before treatment and after 4 cycles of therapy. The data are presented in Table 1. Considering all patients tested, there was no difference in the pretreatment level of $CD4^+$ $foxp3^+$ cells between responders and non-responders (p=0.61). However, post treatment there was a significant increase in the CD4$^+$foxp3$^+$ cells in responding patients (16.87 ± 2.26%) compared to non-responders (11.08 ± 1.01%) (p=0.02). The increase in CD4+foxp3+ cells (post treatment minus pre treatment) was also significantly greater in the responding patients compared to non-responders (p =0.01).

When considering the CD4$^+$foxp3$^+$ cells separately in the individual randomized cohorts, the numbers of patients became small (11 in the IL-2 arm and 22 in the IL-2 plus vaccine arm) (See Table 1). In the IL-2 arm, there was no difference in the pre treatment levels of these cells comparing responders to non-responders, however, there was a trend towards increased levels of CD4$^+$ foxp3$^+$ cells in the post treatment samples comparing responders to non-responders (0=0.07). In the patients receiving IL-2 plus vaccine there was no difference in the values of the responding patients compared to the non-responders. Thus, the increase in CD4$^+$foxp3$^+$ cells post treatment in responding patients was likely related to IL-2 and not the vaccine.

Table 1: In-vitro analysis of circulating suppressor cells

	Percent foxp3$^+$ cells in CD4$^+$ PBMC		
Responders	(n) Pre treatment	(n) Post treatment	(n) Difference (Post - Pre)
<u>All</u>			
Yes	(11) 7.33 ± 0.90	(12) 16.87 ± 2.26	(11) 9.81 ± 2.20
No	(22) 7.92 ± 0.67	(23) 11.08 ± 1.01	(22) 3.12 ± 0.97
	p* =0.61	p=0.02	p=0.01
<u>IL-2 alone</u>			
Yes	(3) 10.40 ± 1.50	(4) 19.43 ± 3.45	(3) 10.87 ± 5.16
No	(7) 7.13 ± 1.26	(7) 11.53 ± 2.10	(7) 4.40 ± 1.52
	p=0.17	p=0.07	p=0.17
<u>IL-2 + Vaccine</u>			
Yes	(8) 6.18 ± .082	(8) 15.59 ± 2.96	(8) 9.41 ± 2.56
No	(15) 8.29 ± 0.81	(16) 10.89 ± 1.17	(15) 2.52 ± 1.24
	p=0.11	p=0.19	p=0.03

* Wilcoxon test

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